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PRINCIPAL INVESTIGATOR: Douglas S. Lyles, Ph.D.

CONTRACTING ORGANIZATION: Wake Forest University School of
Medicine
Winston-Salem, North Carolina 27157

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Table of Contents

Cover.....	1
SF 298.....	2
Table of Contents.....	3
Introduction.....	4
Body.....	4
Key Research Accomplishments.....	14
Reportable Outcomes.....	15
Conclusions.....	15
References.....	16
Appendices.....	

Introduction:

The goal of this project is to develop novel vectors for therapy of prostate tumors based on vesicular stomatitis virus (VSV). VSV kills many tumor cells more effectively than normal cells, due in part to defects in the antiviral response in tumor cells. The novelty in our approach is our ability to enhance the selectivity of killing of tumor cells versus normal cells by manipulating the viral genes that control the antiviral interferon response. Aim 1 is to identify mutations in VSV genes that enhance the differential killing of prostate tumor cells versus normal cells. Aim 2 is to identify VSV mutants that enhance the antiviral interferon response in prostate cells. Aim 3 is to determine whether VSV mutants have greater efficacy and safety than wild-type VSV in reducing prostate tumors in nude mice. In the previous reporting period, we had largely completed the experiments in Aims 1 and 2 for normal prostate cells and the tumor cells (LNCaP and PC-3), and identified several mutant viruses with the desired properties. In addition, we had preliminary data on Aim 3, indicating that one of the mutant viruses is an effective, and safer vector for treating LNCaP tumors in nude mice. During this reporting period we completed the *in vivo* experiments with LNCaP tumors and submitted a manuscript to the *Journal of Virology* describing our results with LNCaP cells. The reviewers of this manuscript requested several additional experiments that primarily address issues of virus growth in prostate cells in culture and pathogenesis of the mutant viruses in mice. These experiments have been completed as described below, and the manuscript is being prepared for resubmission. In addition, during the present reporting period, we have begun our experiments with tumor cells cultured from patient prostatectomy specimens and the *in vivo* experiments with the more resistant PC3 tumor cell line.

Body:

Aim 1: To identify mutations in VSV genes that enhance the differential killing of prostate tumor cells versus normal cells.

Task 1: Determine rate of induction of apoptosis in tumor cells versus normal cells by time-lapse video microscopy (months 1-9)

- a. Establish secondary cultures of prostate tumor cells and normal prostatic epithelial cells from two different patients. Tumor cell lines LNCaP and PC3 will be continuously maintained throughout the project.*
- b. Infect cells and collect video microscopy data from infection of six different cell cultures (normal and tumor cells from two different patients, LNCaP cells, PC3 cells) with four different viruses (two wild-type and three mutant VSV strains).*
- c. Repeat experiments 3-5 times until statistical significance of differences in the results can be established.*

Accomplishments for task 1:

This task as described in the original proposal was completed during the previous reporting period. As suggested in the review of the previous report, we here describe the VSV strains we tested in more detail. We tested five different viruses for their ability to infect and kill tumor and benign prostate cells. These experiments were done to show which mutations enhance

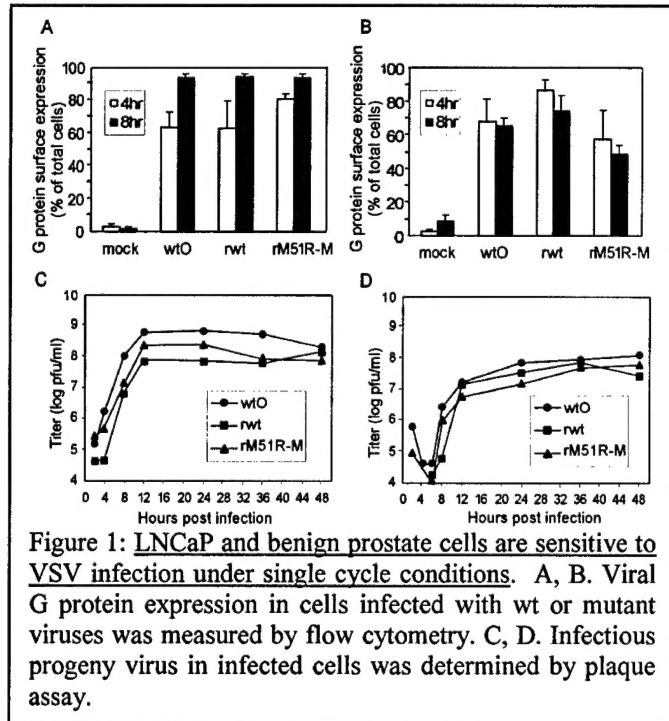
differential killing of tumor versus normal cells by VSV. Wild-type (wt) VSV strains included the naturally occurring Orsay strain (wtO), which has been studied in research laboratories for many years, and a recombinant wild-type (rwt) virus derived from an infectious cDNA clone modified slightly(6) from the one described by Whelan et al. (9). This virus has genes derived from several different strains of VSV. The mutant viruses contained point mutations in the M protein. The matrix (M) protein of VSV serves two major classes of functions in virus-infected cells (reviewed in (7)). One class of functions are involved in assembly of progeny virions. The other class of functions is involved in suppressing host gene expression, particularly the induction of antiviral responses. We have shown previously that a point mutation substituting methionine for arginine at position 51 of the 229 amino acid M protein (M51R mutation) renders M protein defective in its ability to suppress host antiviral responses, but the mutant M protein is as effective as wt M protein in the virus assembly functions necessary for virus replication (1-3, 5). RM51R-M virus is a recombinant virus that is isogenic with rwt virus except for the M51R mutation. The r1026-M virus and tsO82 virus are similar M51R-M protein mutants on different strain backgrounds (2). R1026 virus is a recombinant virus that is isogenic with rM51R-M virus, except the M protein is derived from the HR strain, and tsO82 virus is a naturally occurring M51R M protein mutant derived from wtO virus. These viruses have somewhat different growth properties and abilities to induce cell death by apoptosis, so we tested the two wt and three M protein mutants for their ability to infect and kill tumor and benign prostate cells. As a result of the experiments in task 1, we chose to focus on two viruses: the rwt and rM51R-M viruses. In some experiments we included wtO as a representative naturally occurring virus, which replicates to higher titers than the recombinant viruses.

Our hypothesis was that wt viruses should infect and kill both tumor and benign cells, whereas M protein mutants should only infect and kill tumor cells. In principle, the antiviral responses induced by the M protein mutant viruses should protect benign cells, but the tumor cells should be susceptible to these viruses because they are defective in their antiviral responses. Our initial experiments in task 1 used time-lapse microscopy to assay the induction of apoptosis by morphological changes in infected cells. These experiments were suggestive, but did not fully support our hypothesis, as pointed out by the reviewer of the previous report. Therefore, during the present reporting period, we used other assays for cell death to test this hypothesis. In addition, in response to the review of our manuscript, we performed experiments to test the effect of single-cycle versus multiple-cycle infection on virus yield and cell death. We also extended these experiments to a second strain of benign prostatic cells. These results have served to strengthen our conclusions. The experiments performed in the present reporting period that represent extensions of task 1 are summarized here.

LNCaP and benign prostate cells are susceptible to infection with wt and mutant viruses under single cycle infection conditions. To determine the effectiveness of M protein mutant viruses as oncolytic viruses for prostate tumor therapy, we tested the ability rM51R-M virus to infect LNCaP tumor cells as well as benign human prostatic epithelial cells in single cycle infection experiments. LNCaP cells and primary cultures of benign prostate cells obtained from patients were infected with wt and mutant viruses and were analyzed for VSV antigen expression, production of infectious viral progeny, and the ability of viruses kill infected cells (Fig. 1 and 2).

The efficiency of infection with wt and mutant viruses was measured by determining the

percentage of cells expressing the viral G protein surface antigen by flow cytometry. LNCaP cells and benign prostatic cells (WFU55PZ strain) were infected with wt and rM51R-M viruses at a multiplicity of 10pfu/cell, so that in theory nearly 100% of cells were infected. At 4 and 8 hr postinfection, cells were fixed and incubated with antibody to G protein and a fluorescein-conjugated secondary antibody, then analyzed by flow cytometry. Results in Fig. 1A show that between 90 and 100% of LNCaP cells infected with wtO, rwt, and rM51R-M viruses expressed



G protein at the cell surface by 8 h post-infection. These data indicate that there is no difference between rM51R-M virus and the wt viruses in their efficiency of infection of LNCaP tumor cells. Similarly, 70-90% of WFU55PZ cells infected with wtO or rwt viruses expressed the viral G protein. WFU55PZ cells infected with rM51R-M expressed G protein to a slightly lesser extent (50-60% of cells) than cells infected with wtO and rwt viruses (Fig. 1B) although this difference was not statistically significant. These data indicate that LNCaP cells and benign prostatic cells are similar in their susceptibility to VSV infection, and that there is little if any difference between viruses with wt and mutant M proteins in their ability to infect these cells.

The ability of wt and M protein mutant viruses to produce infectious progeny in LNCaP and WFU55PZ cells was determined by plaque assay (Fig. 1C and D). In LNCaP cells, wtO virus grew to titers approaching 10^9 pfu/ml, similar to results with other susceptible cell lines. Rwt virus grew to approximately one log lower titers, which is also similar to results in other cell types (2, 6). Variability between titers in repeated experiments was less than half a log. Thus, this difference between naturally occurring and recombinant viruses was statistically significant at most time points. RM51R-M virus grew to titers intermediate between those of rwt and wtO viruses, indicating that there is no defect in virus growth resulting from the M protein mutation. All three viruses produced similar levels of infectious progeny in WFU55PZ cells (Fig. 1D), although the titers of rM51R-M virus were slightly less than those of rwt virus at most times post-infection. Despite the difference between LNCaP and WFU55PZ cells in virus titers, these data indicate that both types of cells are permissive for virus replication.

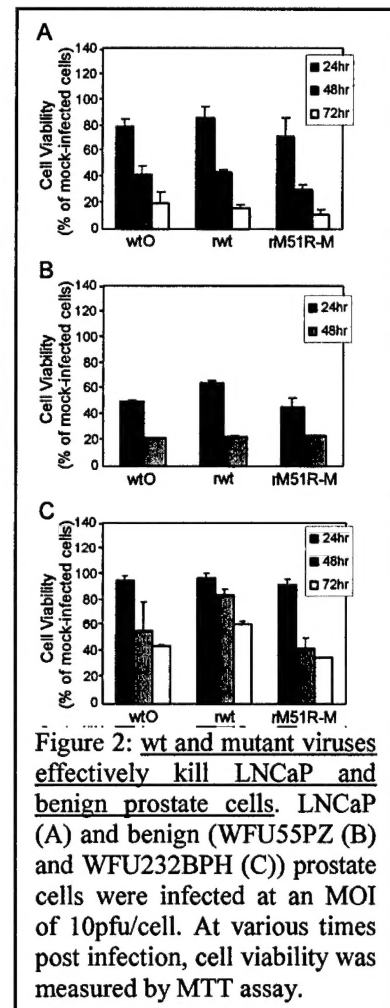
The ability of wt and M protein mutant viruses to kill prostate tumor and benign cells was determined by MTT assay, which determines the metabolic activity of viable cells. These experiments compared LNCaP and WFU55PZ cells, and another benign prostate cell strain isolated from a patient with benign prostatic hyperplasia, WFU232BPH. Cells were infected at an MOI of 10 pfu/cell with each of the viruses and cell viability was measured at different times postinfection (Fig. 2). Results show that LNCaP cells were sensitive to killing by wt and mutant viruses and that there was no difference between wt and mutant viruses in their ability to kill

these cells (Fig. 2). Similarly, WFU55PZ benign prostatic epithelial cells were sensitive to killing by each of the viruses so that by 48 h postinfection, approximately 20% of infected cells remained viable (Fig. 2B). WFU232BPH cells were somewhat more resistant to virus-induced cell killing, with approximately 40% of cells remaining viable at 72 h postinfection (Fig. 2C). Despite this difference between prostate cells from different patients, we can conclude from figures 1 and 2 that both prostate tumor and benign cells are susceptible to infection with wt and mutant viruses under single cycle infection conditions, and that M protein does not play a significant role in VSV-induced cell killing in the prostate. Further studies indicated that there was no difference between LNCaP and benign prostate cells in their susceptibility to VSV-induced apoptosis as measured by TUNEL assay and that rM51R-M virus was as effective as wt viruses at inducing apoptosis these cells in single cycle infections (data not shown).

Benign prostate cells are more resistant to infection with M51R-M virus than wt viruses in multiple cycle infection experiments.

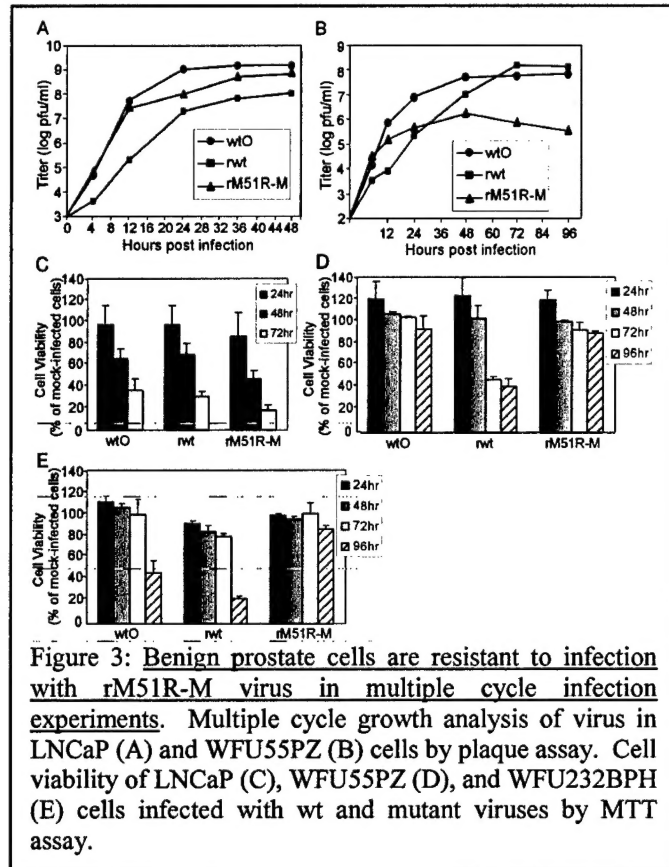
To determine whether antiviral responses affect spread of virus to surrounding cells, we tested the ability of wt and mutant viruses to infect LNCaP and benign tumor cells under multiple cycle infection conditions. We used a multiplicity of infection of 0.1pfu/cell, so that approximately 10% of cells were initially infected, and growth of virus depends on spread to surrounding uninfected cells. Similar to data obtained under single cycle infection conditions, rM51R-M grew to titers intermediate between those of rwt and wtO viruses in LNCaP cells in the multiple cycle growth experiment (Fig. 3A). Although all viruses reached titers as high as those observed in the single cycle growth experiment (Fig. 1C), there was a delay in growth due to differences in kinetics of virus spread. This delay was also seen in WFU55PZ cells infected with each of the viruses (Fig. 3B). However, in contrast to LNCaP cells, rM51R-M virus produced lower levels of infectious virus progeny than wt viruses in WFU55PZ cells. In fact, by 96 h post-infection, rM51R-M virus titers were greater than two logs lower than those of rwt and wtO viruses. Similar data were obtained from WFU232BPH benign prostate cells (data not shown). These data suggest that the elevated antiviral response in benign prostate cells infected with rM51R-M virus limits virus spread to surrounding cells.

The ability of wt and mutant viruses to kill LNCaP and benign prostate cells under multiple cycle infection conditions was measured by MTT assay. Data indicate that LNCaP cells are equally susceptible to killing by each of the viruses (Fig. 3C). However, both isolates of benign prostate cells were highly resistant to cell killing by rM51R-M virus. Close to 90% of benign prostate cells infected with rM51R-M virus retained viability at 96 h post-infection (Fig. 3D and E). In contrast, both cell strains were sensitive to killing by rwt virus. WFU232BPH, but not WFU55PZ cells, were also sensitive to killing by wtO virus. In contrast to data obtained in the single cycle experiments, primary cultures of benign prostate



cells are consistently resistant to infection with rM51R-M virus in multiple cycle infection experiments, likely due to the ability of this virus to induce an effective antiviral response in these cells as compared to LNCaP cells. The observation that WFU55PZ cells were also resistant to wtO virus infection at low multiplicity of infections suggests that these cells are also

able to mount an antiviral response against wtO virus.



To summarize the results of these cell culture experiments, these data show that neither benign nor tumor prostate cells are inherently resistant to VSV infection and killing in single-cycle experiments. Furthermore, the M protein mutation had little if any effect on the ability of VSV to induce apoptosis in prostate cells. In contrast to single-cycle infection, multiple-cycle replication of the M protein mutant virus was severely restricted in benign prostate cells, but not in tumor cells. Thus the M protein mutant virus showed a greater selectivity for tumor cells versus normal cells than its wt control under conditions of multiple-cycle virus growth.

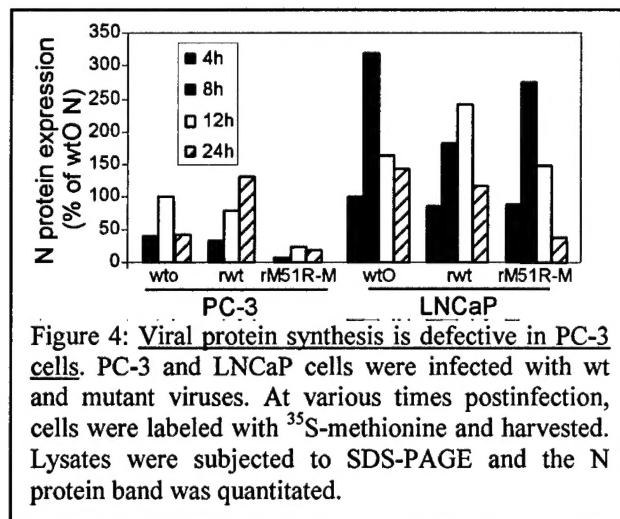
Task 2: Determine the activation of caspase 3 in tumor cells versus normal cells infected with wild-type and mutant strains of VSV (months 3-12).

- Infect cells for varying periods of time and prepare cells extracts from infection of six different cultures with four different viruses as in Task 1. The time points will be chosen based on the time-lapse data obtained in Task 1.
- Assay caspase 3 activity using a fluorogenic substrate.
- Repeat experiments 3-5 times until statistical significance of differences in the results can be established.

This task was completed during the previous reporting period, and the results are described in the previous report. A brief description of these results follows:

Human prostate cancer cell lines differ in their sensitivity to VSV infection. The main idea governing the selectivity of anti-tumor therapies using VSV is that many tumor cell lines are unresponsive to the anti-proliferative effects of IFN and should be correspondingly susceptible to VSV infection. However, we have found that human prostate cancer cell lines vary in their susceptibilities to VSV infection as well as their responsiveness to IFN. We compared the efficiency of infection of LNCaP cells as well as another prostate tumor cell line, PC-3, with wt and mutant viruses. The efficiency of infection was measured by determining the percentage of

cells expressing the viral G protein surface antigen by flow cytometry. Between 90 and 100% of LNCaP cells infected with wtO, rwt, and rM51R-M viruses expressed G protein at the cell surface by 8 h post-infection. However, in contrast, PC-3 cells were highly resistant to infection with VSV, with only 10-30% of cells expressing the viral G protein. To determine whether the degree of infectivity correlated with VSV-induced apoptosis, LNCaP and PC-3 cells were infected with wtO, rwt and rM51R-M viruses and cells were analyzed for caspase-3 enzymatic activity. Results clearly showed that VSV-infected LNCaP cells were apoptotic as indicated by high caspase-3 activity while PC-3 cells had very low levels of caspase-3 activity. Taken together, the data indicate that PC-3 cells are resistant to infection with VSV while LNCaP cells are highly sensitive to VSV-induced cell killing, and that there is little if any difference between viruses with wt and mutant M proteins in their ability to infect these cells.



In the present reporting period, we extended these experiments to analyze the steps in the virus replication cycle that may be delayed or altered in VSV-resistant cells. Figure 4 shows that the rates of viral protein synthesis are lower in PC-3 cells as compared to LNCaP cells. For this experiment, cells were infected with wt and mutant viruses at a multiplicity of 10pfu/cell. At various times post infection, cells were incubated with ³⁵S-methionine for 15 min, harvested and lysed. Lysates were subjected to SDS-PAGE and the labeled viral nucleocapsid (N) protein was quantitated by phosphorimaging. N protein

expression is expressed as a percentage of the wtO N protein at 4 h for LNCaP cells and at 12 h for PC-3 cells, at which time equivalent levels of protein were observed for LNCaP and PC-3 cells. Results indicate that viral protein synthesis in VSV-sensitive LNCaP cells reaches a peak at 8 h, after which rates decrease due to VSV-induced cytopathic effect. The rates of protein synthesis in LNCaP cells are similar to those observed in cells displaying signs of robust VSV infection, such as HeLa and BHK cells. However, the rates of N protein synthesis in PC-3 cells are much lower than those in LNCaP cells. These results suggest that the antiviral response serves to inhibit viral protein synthesis in PC-3 cells.

Main conclusions from Aim 1:

1. Neither benign nor tumor prostate cells are inherently resistant to VSV infection and killing in single-cycle experiments.
2. The M protein mutation had little if any effect on the ability of VSV to induce apoptosis in prostate cells.
3. In contrast to single-cycle infection, multiple-cycle replication of the M protein mutant virus was severely restricted in benign prostate cells, but not in LNCaP tumor cells. Thus the M protein mutant virus showed a greater selectivity for tumor cells versus normal cells than its wt control under conditions of multiple-cycle virus growth.

4. LNCaP and PC-3 prostate tumor cells are differentially susceptible to infection by wt and mutant viruses. LNCaP cells are extremely sensitive to virus infection and killing, whereas PC-3 cells are relatively resistant.

Work to be done for Aim 1:

1. We will extend these experiments to include secondary cultures of prostate tumor cells and determine the ability of each of the viruses to infect and kill these cells. This would allow us to determine which cell type (LNCaP or PC-3), most closely resembles prostate tumor cells from patients. We have preliminary data on one such cell strain, which appears to be intermediate in its sensitivity between LNCaP and PC3 cells.

Aim 2: To identify mutations in VSV genes that enhance the antiviral interferon response in normal prostatic epithelial cells versus prostate tumor cells.

Task 3: Determine the level of interferon production by tumor cells versus normal cells by ELISA assay (months 13-18).

- a. Conduct preliminary experiments by stimulating cells with poly I:C to determine whether the cells produce alpha or beta interferons using the appropriate ELISA assay.
- b. Infect cells and collect culture supernatants from infection of six different cell cultures (similar to Aim 1) with six different viruses (the same four viruses used in Aim 1, plus an additional interferon-inducing mutant and its corresponding wild-type strain). Assay interferon levels by ELISA assay.
- c. Repeat experiments 3-5 times until statistical significance of differences in the results can be established.

Accomplishments for task 3:

This task was completed in the previous reporting period and the results were incorporated into a manuscript that was published during the previous reporting period. The reviewer of the previous report had an editorial comment about how we expressed the goals of this part of the project. A main goal of this project is to develop viruses that have a high degree of selectivity for infecting tumor cells and not normal cells. At the point where these viruses may be used in cancer patients, this greater selectivity will result in viruses that are safer for use in patients, by virtue of their inability to replicate in normal tissue, but these viruses should still retain their effectiveness for replication and killing in tumor tissue. Our hypothesis is that M protein mutant viruses that induce interferon would be safer vectors due to their ability to induce an effective antiviral response in normal cells. To test this *in vitro*, we determined the level of interferon production by tumor cells versus normal cells by an interferon bioassay. The results showed that interferon activity was detected in each of these cell lines infected with the M protein mutant viruses (tsO82, rM51R and r1026) to varying degrees. Our data indicate that rM51R virus was the most effective inducer of interferon.

Task 4: Determine responsiveness of normal versus tumor cells to exogenously added interferons (months 16-24)

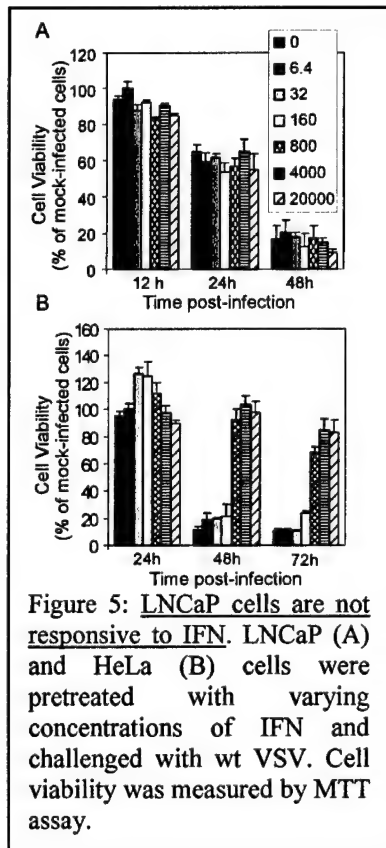
- a. Treat cell cultures with interferon prior to infection with wild-type or mutant strains of

VSV. The choice of interferons to use in these experiments will be based on the results of Task 3. Six different cell cultures and four different viruses will be used in these experiments, as in Task 1.

b. Collect time lapse microscopy data to determine the effect of interferon treatment on cell killing by VSV.

c. Determine virus yields from treated cultures by plaque assays.

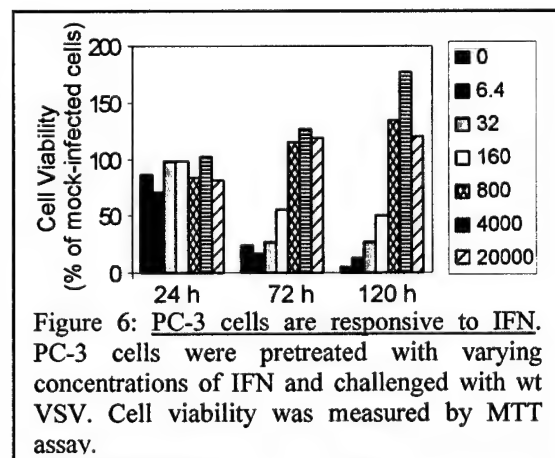
d. Repeat experiments 3-5 times until statistical significance of differences in the results can be established.



Accomplishments for task 4:

LNCaP prostate tumor cells are unresponsive to IFN. Clinical studies indicate that metastatic prostate tumors are not responsive to IFN treatments. (4, 8) Figure 5 illustrates this lack of responsiveness using the LNCaP cell line. LNCaP cells were incubated with varying concentrations of IFN for 16h and challenged with VSV. At different times postinfection, cell viability was measured by MTT assay. Cells responsive to IFN should exhibit an IFN dose-dependent resistance to virus infection, while cells that are not responsive to IFN should show no difference in cytopathic effect regardless of IFN concentration. HeLa cells were used as a control since they are known to respond to IFN. Data in figure 5A show a decrease in LNCaP cell viability over the time course of the experiment due to virus infection. Furthermore, the results indicate that LNCaP cells are not responsive to IFN since they were not protected from VSV infection by pretreatment with IFN concentrations as high as 20,000 IU/ml. In contrast, HeLa cells were responsive to IFN (Fig. 5B) since close to 100% of cells displayed resistance to VSV infection upon pretreatment with concentrations of IFN ≥ 800 IU/ml.

In a separate series of experiments, we tested the responsiveness of PC-3 cells to IFN, PC-3 cells were incubated with varying concentrations of IFN for 16h and challenged with VSV at an MOI of 50pfu/cell. At different times postinfection, cell viability was measured by MTT assay. First of all, Figure 6 shows that PC-3 cells were resistant to VSV infection since all cells remained viable by 24 h postinfection. However, they were also responsive to IFN since close to 100% of cells displayed resistance to VSV infection upon pretreatment with concentrations of IFN ≥ 800 IU/ml by 72 h postinfection.



Main conclusions from Aim 2:

1. M protein mutant viruses induce interferon in normal prostate, PC-3 and LNCaP cells, whereas wt viruses do not.
2. LNCaP and PC-3 cells are differentially responsive to interferon. LNCaP cells, similar to many tumor cells, are not responsive to interferon. PC-3 cells are responsive to interferon.
3. The M protein mutant virus, rM51R, induces the highest levels of interferon in normal prostate cells.

Work to be done for Aim 2:

1. Determine the responsiveness of secondary cultures of normal prostate cells and prostate tumor cells to interferon.

Aim 3: To determine whether VSV mutants have greater efficacy and/or safety than wt VSV in reducing prostate tumors in nude mice.

Task 5: Establish tumors in nude mice and treat tumors with wild-type and mutant strains of VSV (months 25-27).

- a. Inject 50 animals each with LNCaP or PC3 cells in order to achieve approximately 40 tumor-bearing animals of each tumor type (100 animals total, 80 final).
- b. Inject tumors with one wild-type strain and two mutant strains of VSV or inject with culture medium as a negative control (10 mice in each group). Measure tumor size and health status of the mice daily. Monitor twice daily if symptoms of VSV infection or tumor burden are apparent.
- c. Euthanize mice that show signs of end-stage illness and harvest tumors and tissues for analysis.

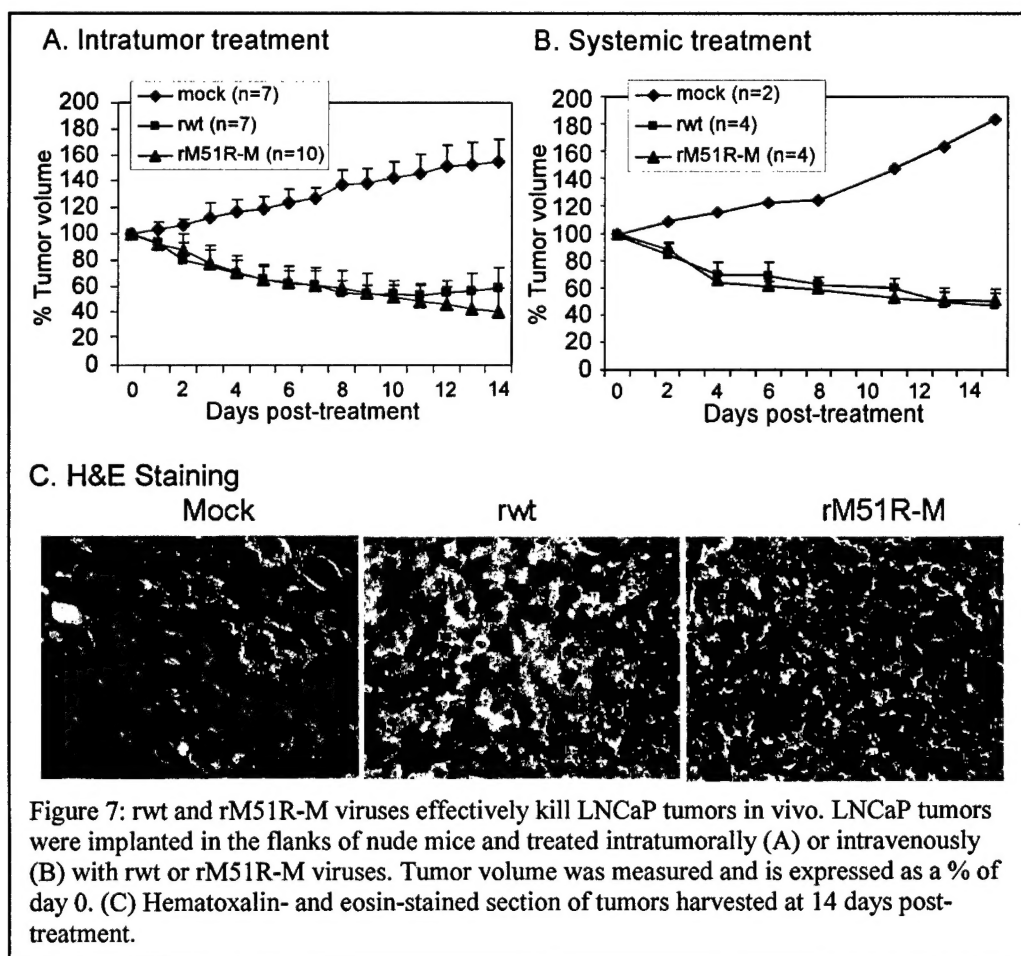
Task 6: Analyze tumors and tissues from treated mice for the presence of virus and induction of apoptosis (months 28-36).

- a. Determine virus titers in tumors and tissues from treated mice by plaque assays. Tumors and four different normal tissues will be examined (brain, lungs, spleen, and blood).
- b. Determine viral antigen expression in tumors and tissues from treated mice by immunohistochemistry.
- c. Determine induction of apoptosis in tumors and tissues from treated mice by TUNEL assay.

Accomplishments for tasks 5 and 6:

Rwt and rM51R-M viruses effectively kill tumor cells *in vivo*. To determine the ability of wt and mutant viruses to kill tumor cells *in vivo*, LNCaP prostate tumor cells were injected subcutaneously in the flanks of BALB/c nude mice. When palpable tumors were obtained, rwt and rM51R-M viruses (10^8 pfu) were injected intratumorally, and tumor volume was measured daily. Results in figure 7A show that tumor volume decreased in animals treated with both rwt

and rM51R viruses, while mock-treated tumors continued to increase in size. By two weeks post-treatment, the tumors of mice injected with rM51R-M virus had decreased to approximately 30% of their initial volume at the start of the experiment. Interestingly, although the tumors of mice treated with rwt virus also regressed, some tumors started to increase in size again by ten days post-treatment. Although there was little if any difference between rM51R-M and rwt viruses in their effects on tumors, there was a striking difference in the morbidity of treated mice. 71% of mice treated with the rwt virus showed severe symptoms of VSV infection, including paralysis, weight loss and disorientation and were sacrificed between days 7 and 14. However, none of the mice treated with rM51R-M virus showed signs of illness and there was complete elimination of tumors by 6 to 8 weeks in some cases. These data indicate that rM51R-M virus is at least as effective as rwt virus in reducing LNCaP tumors in vivo when administered intratumorally and support our hypothesis that M protein mutant viruses are safer viruses for anti-tumor therapies due to their ability to induce antiviral responses and spare normal cells. In fact, the M mutant virus is also as effective as rwt virus at killing prostate tumor xenografts when administered systemically (Fig. 7B), further supporting the data that this virus is able to spread and replicate in vivo.



Histological examination of tumors by hematoxylin and eosin (H&E) staining at day 14 postinfection showed that mock-infected tumor cells had well defined cell borders and hyperchromatic nuclei (Fig. 7C). The cytoplasm of these cells was vesicular and eosinophilic,

with evidence of mitoses. In contrast, the tumors treated with rwt and rM51R-M viruses were extensively necrotic, characterized by loss of nuclear staining, increased cytoplasmic eosinophilia, and loss of cellular detail and cell borders. The additional tissue eosinophilia in tumors infected with rM51R-M virus was due to infiltrating erythrocytes as a result of hemorrhaging. These data support the idea that the remaining tumor mass at day 14 consisted largely of dead or dying tumor cells.

Main conclusions from Aim 3:

1. Both rwt and rM51R viruses are effective killers of tumor cells *in vivo*.

Work to be done for Aim 3:

1. Tissue (tumor, brain, spleen, lung and liver) harvested from LNCaP tumor bearing mice and processed for immunohistochemistry and viral titer determination
 - a. Determine virus titers in tissues to determine spread of virus
 - b. Determine viral antigen expression in treated tumors (using antibodies to G surface glycoprotein of virus)
 - c. Tissue will be stained with caspase 3 antibody, or TUNEL to determine apoptosis in tumor and other tissue infected with virus
2. Nude mice will be implanted with PC-3 tumor cells to determine the ability of rwt and rM51R viruses to kill this different type of tumor cells *in vivo*.
3. Tissue from PC-3 tumor bearing mice will be harvested and processed for immunohistochemistry and viral titer determination as in #1.
4. We will also test the M protein mutant virus, r1026, in a preliminary study to determine its effectiveness an anti-tumor vector.

Key research accomplishments:

1. M protein mutant viruses are effective killers of prostate tumor cells.
2. M protein mutant viruses effectively induce interferon in normal prostate cells and tumor cells.
3. LNCaP and PC-3 prostate tumor cells are differentially responsive to interferon. LNCaP tumor cells are not responsive to interferon, while PC-3 cells are responsive.
4. We have identified an M protein mutant virus, rM51R, as a potential vector for tumor therapy. This virus effectively kills tumor cells and induces high levels of interferon in normal prostate cells.
5. Both rwt and rM51R viruses are effective killers of tumors in nude mice. However, most of the mice treated with rwt virus succumb to virus infection, while mice treated with rM51R-M virus show no signs of virus-induced illness.
6. The difference in cell killing and responsiveness to interferon between LNCaP and PC-3 cells will be exploited to determine which naturally-occurring tumors are likely to be responsive to oncolytic virus therapy with VSV.

Reportable outcomes:

1. We are currently preparing a manuscript for resubmission, based on the new data obtained in Aim 1.

Conclusions:

Aim 1 of this proposal was to identify mutations in VSV genes that enhance the differential killing of prostate tumor cells versus normal cells. The results from our study indicated that the M protein mutant viruses effectively killed prostate cells *in vitro*. LNCaP tumor cells and benign prostatic cells were similarly sensitive to virus infection in single-cycle infection experiments, indicating that normal prostatic cells are not inherently resistant to virus infection. In multiple-cycle infection experiments, benign prostatic cells were resistant to infection with the M protein mutant virus (but not wild-type virus), due to the induction of antiviral responses by the mutant virus. In contrast, LNCaP tumor cells were effectively killed by the M protein mutant virus in both single-cycle and multiple-cycle experiments, due to their defective antiviral responses. These results support our basic hypothesis that M protein mutant viruses will show a greater selectivity for killing tumor cells versus normal cells. Furthermore, in Aim 1 we identified a tumor cell line that is less susceptible to virus infection (PC-3). We are interested in the differences between the PC3 and LNCaP cell lines since identifying these differences may allow us to predict which prostate tumors are likely to respond to oncolytic virus therapy with VSV.

Aim 2 was to identify VSV mutants that enhance the antiviral interferon response in prostate cells. We found that the M protein mutant viruses induced interferon in both normal prostate cells and prostate tumor cells. In addition, PC-3 and LNCaP cells were differentially responsive to interferon. Because of the differences between these two cell types, it will become important to establish secondary cultures of prostate tumors to determine which tumor cells line resembles prostate tumors in patients. Aim 3 was to determine whether VSV mutants have greater efficacy and safety than wild-type VSV in reducing prostate tumors in nude mice. Both rwt and rM51R viruses are effective killers of LNCaP tumors in nude mice. However, most of the animals treated with rwt virus also succumbed to virus infection within 4 weeks post-treatment. In contrast, animals treated with rM51R virus eliminated the tumor and remained healthy for 2 months post-treatment.

We did not digress greatly from our current plan in this proposal. We feel that once we have completed the gaps in the *in vitro* work, including the data from secondary cultures of tumor cells, as well as the animal work in Aim 3, we will be in a good position to judge whether our M protein viruses can be used for effective and safe tumor therapies in primates, and clinical trials within 5 years. There are compelling reasons for developing new treatments for prostate cancer, including the development of viral vectors. Approximately 1 out of every 10 men will develop this form of cancer, and it is second only to lung cancer as the leading cause of cancer death in men. Each year more than 30,000 deaths are caused by this disease. Current treatment of tumors that remain confined to the prostate gland is usually successful, with a 5-year survival rate of 88%. The challenge to develop novel therapies for prostate tumors is in the treatment of metastatic tumors that have spread to many other sites in the body or to areas that are difficult to access. The 5-year survival rate for patients with metastatic prostate cancer is only 29%. The use

of viruses that have been genetically engineered to kill tumor cells offers a promising approach to the treatment of metastatic cancer, because of the natural ability of viruses to spread throughout the body and seek out the tissues that are susceptible to infection. Our mutant viruses have great potential to be used in such therapies due to their added features of safety and of targeting tumor cells due to inherent cellular defects in the interferon pathway.

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Personnel receiving pay from research effort:

1. Douglas S. Lyles, Ph.D.
2. Maryam Ahmed, Ph.D.
3. Scott D. Cramer, Ph.D.
4. Leanne N. Thomas
5. April Coan